Generation of a stable transformant of aldosterone synthase gene (CYP11B2) promoter, and analyses of the effects of angiotensin II receptor blockers (ARBs) on the CYP11B2 expression

博　士　論　文

Generation of a stable transformant of aldosterone synthase gene (*CYP11B2)* promoter, and analyses of the effects of angiotensin II receptor blockers (ARBs) on the *CYP11B2* expression

（アルドステロン合成酵素遺伝子（*CYP11B2*）プロモーター安定発現株の作成および、それを用いてのアンジオテンシン II 受容体拮抗薬による *CYP11B2* 発現調節の検討）

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SUMMARY

Aldosterone synthase gene (*CYP11B2*) encodes the key enzyme of adrenal aldosterone production. I here generated a stable H295R cell line expressing *CYP11B2* 5'-flanking region/luciferase cDNA chimeric construct that is highly sensitive to angiotensin II (AII) and KCl comparing with the transient transfection using the same promoter region. I then examined the effects of various AII receptor blockers (ARBs) on *CYP11B2* transcription using the cell line. In the presence of AII, all ARBs suppressed the AII-induced *CYP11B2* transcription activation. However, telmisartan, but not other ARBs, increased *CYP11B2* transcription in the absence of AII. Telmisartan increased *CYP11B2* transcription dose-dependently and maximally at 12 h. Additionally, telmisartan was observed to increase *CYP11B2* mRNA expression dose-dependently and maximally at 12 h. Moreover, telmisartan dose-dependently increased aldosterone secretion. Transient transfection experiments using *CYP11B2* 5'-flanking region deletion mutants/Ad5 point mutant indicated that the Ad5 element was responsible for the telmisartan effect. Examinations of transcription factors known to bind to the Ad5 element revealed that telmisartan significantly induced nerve growth factor-induced clone B (NGFIB) and Nur-related factor 1 (NURR1) expression maximal at 1 h and gradually decreased steroidogenic factor-1 expression. The telmisartan effect on *CYP11B2* transcription/mRNA expression was abrogated by KN-93, a

Ca^{2+}/calmodulin-dependent kinase (CaMK) inhibitor, treatment. Interestingly, the telmisartan-induced NURR1 mRNA expression, but not NGFIB mRNA expression, was also abrogated by KN-93. Additionally, the over-expression of NURR1 significantly augmented the telmisartan-mediated *CYP11B2* transcriptional activation. Interestingly, the telmisartan-mediated *CYP11B2* transcription was not affected by high-dose olmesartan treatment. Taken together, telmisartan may stimulate *CYP11B2* transcription via the CaMK-mediated induction of NURR1 that activate the Ad5 element, and AII type 1 receptor may not be involved in the effect.

INTRODUCTION

Hypertension is well known as one of the most important risk factors for the progression of atherosclerosis, which may result in the induction of myocardial infarction, brain infarction/hemorrhage, and chronic kidney disease. In 2000, the number of patients with hypertension in the world is speculated as one-quarter among the whole adult population, and is considered to be increasing every year.[1] Among hypertensive patients, approximately 20~30% are estimated as “resistant hypertension” who are above their target blood pressure even in the simultaneous use of 3 anti-hypertensive drugs in different classes.[2] Since the renin-angiotensin-aldosterone system (RAAS) is the main humoral pathway that are profoundly involved in the etiology of hypertension, anti-hypertensive drugs blocking the RAAS including the direct renin inhibitor, angiotensin-converting enzyme inhibitors, angiotensin II (AII) receptor blockers (ARBs), and aldosterone blockers have been clinically used so far.[3] However, in order to prevent the increase of patients with “resistant hypertension,” innovation of novel drugs with different mechanism(s) than the pre-existing RAAS blockers may possibly be necessary.

Aldosterone synthase gene (*CYP11B2*) encodes the key enzyme of adrenal aldosterone production that is mainly regulated by AII and potassium, but also by corticotropin (ACTH) and cyclic AMP (cAMP)[4), 5)]. Since aldosterone, in combination with sodium, plays an important role in the progression of hypertension and vascular damages,[6)] the innovation of novel drugs that inhibit *CYP11B2* transcriptional activation may be advantageous for the treatment of "resistant hypertension." I therefore have been examining the drug-mediated transcriptional regulation of the gene by transient transfection using human adrenocortical carcinoma H295R cells that are well known to secrete aldosterone as well as cortisol,[7)] and have recently demonstrated that peroxisome proliferator-activated receptor (PPAR)-γ agonist thiazolidinediones inhibit both AII- and potassium-mediated *CYP11B2* transcriptional activation via Ca^{2+}/calmodulin-dependent kinase (CaMK) inhibition.[7)]

For the screening of novel drugs, it is important to augment the sensitivity of *CYP11B2* transcriptional level to be able to detect subtle changes. I therefore generated a stable H295R cell line expressing *CYP11B2* 5'-flanking region/luciferase cDNA chimeric construct that is highly sensitive to AII and potassium comparing with the transient transfection using the same region. In contrast to AII, the effects of ARBs on *CYP11B2* transcriptional regulation remain uncertain. I therefore examined the effects of various ARBs on *CYP11B2* transcription using the stable cell line.

OBJECTIVE

My objective in the present study is to generate a stable H295R cell line expressing *CYP11B2* 5'-flanking region/luciferase cDNA chimeric construct that is sensitive enough for the future innovation/discovery of novel anti-hypertensive drugs that inhibit *CYP11B2* transcription. Using the cell line, I also aimed to examine the effects of various ARBs on *CYP11B2* transcription.

EXPERIMENTAL PROCEDURES

Reagents

Telmisartan, human AII, human ACTH, N6-2'-O-Dibutyladenosine-3',5'-cyclic monophosphate (dbcAMP), and Src Inhibitor-1 (Src-I1) were purchased from Sigma (St. Louis, MO). Olmesartan medoxomil was purchased from Toronto Research Chemicals (North York, Canada). Losartan potassium was purchased from LKT Laboratories (St. Paul, MN). Valsartan was kindly provided by Novartis Pharma (Basel, Switzerland). Hygromycin B was purchased from Nacalai Tesque (Kyoto, Japan). KN-93 and Ro-31-8220 was purchased from Calbiochem (Darmstadt, Germany).

Plasmids

The subcloned chimeric constructs containing the human *CYP11B2* genomic DNA and luciferase cDNA (pGL3-Basic, Promega, Madison, WI) kindly provided by Dr. William E. Rainey (Medical College of Georgia) were used for the transient transfection studies: -1521/+2-luc (harboring the *CYP11B2* 5'-flanking region from -1521 to +2 relative to the transcription start site upstream of the luciferase cDNA in pGL3-Basic), -747/+2-luc; -135/+2-luc; -106/+2-luc; -65/+2-luc. Ad5 element in -1521/+2-luc was mutated from 5'-CTCCAGCCTTGACCTT-3' to

5'-CTCCAGCCTTGAtaTc-3' (-1521/+2-luc-Ad5-mut)[7), 8)] using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). β-galactosidase control plasmid in pCMV (pCMV-β-gal) was purchased from Clontech (Palo Alto, CA). Murine nerve growth factor-induced clone B (NGFIB) and Nur-related factor 1 (NURR1) cDNAs were cloned by PCR from murine pituitary AtT20 cell RNA and subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) (pcDNA3-NGFIB and pcDNA3-NURR1). The newly subcloned chimeric construct containing the human *CYP11B2* 5'-flanking region (-1521 to +2) and luciferase cDNA (pGL4.15, Promega) termed pGL4-*CYP11B2* was used for the generation of a stable cell line.

Cell Culture/Generation of Stable Cell Line

H295R cells were grown with 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), Insulin-Transferrin-Selenium-G Supplements (Invitrogen), 1.25 mg/mL BSA (Sigma), 5.35 µg/mL linoleic acid (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO_2. The stable cell line expressing pGL4-*CYP11B2* was generated as follows. When H295R cells were grown to 80% confluence, they were transfected with pGL4-*CYP11B2* using Lipofectamine™ LTX and Plus reagent

(Invitrogen) for 96 h according to the manufacturer's instructions. Thereafter, the cells were incubated with hygromycin B (200 μg/ml) for the selection, and the stable cell line (*CYP11B2*-H295R) was finally obtained. *CYP11B2*-H295R cells were grown to 100% confluence in either 24- or 96-multiwell plates, and were thereafter exposed to ARB for 12 h in DMEM supplemented with 1% charcoal/resin treated (stripped) FBS.[7)] In case the cells were co-treated with AII, they were exposed to 100 nmol/L AII for the last 6 h. In some experiments, the cells were exposed either to 10.4 mmol/L (16 mEq/L of K^+) potassium (KCl), 10 μmol/L ACTH, 100 nmol/L AII, or 1 mmol/L dbcAMP for 6 h.

RNA Preparation and Quantitative RT-PCR

When H295R cells were grown to 100% confluence in 24-multiwell plates, they were exposed to telmisartan for 12 h in DMEM supplemented with 1% stripped FBS. H295R cell total RNA was extracted with TaKaRa FastPure RNA Kit (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. Total RNAs were subjected to reverse transcription (RT) reaction using PrimeScript Reverse Transcriptase (Takara Bio) with random 6mer and oligo dT primers according to the manufacturer's instructions. Thereafter, obtained templates were used for quantitative real-time PCR (95 °C, 3 min for 1 cycle; 95 °C, 15 sec; 60 °C, 10 sec; 72 °C, 20 sec for 40 cycles) with iQ Supermix (for *CYP11B2*) or iQ SYBR green Supermix (for others) (Bio-Rad, Hercules, CA) by DNA Engine thermal cycler attached to Chromo4 detector (Bio-Rad). The following primers and TaqMan probe (for *CYP11B2*) sequences were used: *CYP11B2* (forward, 5'-GGCAGAGGCAGAGATGCTG-3', reverse, 5'-CTTGAGTTAGTGTCTCCACCAGGA-3', probe, 5'-CTGCACCACGTGCTGAAGCACT-3'), NGFIB（forward, 5'-GGAGTGCACAGAAGAACTTC-3', reverse, 5'-GGCTTGGATACAGGGCATCT-3'）, NURR1（forward, 5'-TGAAGAGAGACGCGGAGAAC-3', reverse, 5'-GAAAGCAATGGGGAGTCCAG-3'）, steroidogenic factor-1 (SF-1) (forward, 5'-AGCAGAAGAAGGCACAGATTCG-3', reverse, 5'-CTGGGAGGCAGCACGTAGTC-3'), β-actin (forward, 5'-CCAACCGCGAGAAGATGACC-3', reverse, 5'-CCAGAGGCGTACAGGGATAG-3').

Measurement of aldosterone concentration

When H295R cells were grown to confluence in 24-multiwell plates, they were exposed to telmisartan for 12 h in DMEM supplemented with 1% stripped FBS.

Aldosterone concentrations of the media were thereafter measured by Aldosterone EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Transient Transfection/Luciferase Assay

When H295R cells were grown to 80% confluence in 24-multiwell plates, they were transiently transfected with 200 ng luciferase reporter plasmids and 100 ng pCMV-β-gal using Lipofectamine LTX and Plus reagent (Invitrogen) for 48 h according to the manufacturer's instructions. In some experiments, expression vector including pcDNA3-NGFIB or pcDNA3-NURR1 (200 ng) was also transfected. The media were changed to DMEM supplemented with 1% stripped FBS, and the cells were incubated with or without telmisartan for 12 h. In some experiments, the cells were exposed either to 10.4 mmol/L KCl, 10 μmol/L ACTH, 100 nmol/L AII, or 1 mmol/L dbcAMP for 6 h. After appropriated treatments, they were washed with PBS, and the cell extracts were prepared using Glo Lysis Buffer (Promega). Luciferase activity was measured using Bright-Glo reagents (Promega), and β-galactosidase activity was simultaneously measured. Data were normalized by β-galactosidase activity. In case of *CYP11B2*-H295R cells (the stable cell line), only luciferase activity was measured after appropriate treatments.

Statistical Analysis

All data are presented as mean ± SEM. Statistical analyses were performed with ANOVA followed by post hoc Tukey test.

RESULTS

Differential regulation of CYP11B2 *transcription between transiently transfected H2965R cells and the stable* CYP11B2-*H295R cells*

I first compared the effects of KCl, ACTH, AII, and dbcAMP on *CYP11B2* transcriptional regulation between transiently transfected H295R cells and the stable *CYP11B2*-H295R cells. In transiently transfected H295R cells, KCl, ACTH, AII, and dbcAMP induced 8.5-fold, 1.9-fold, 5.7-fold, and 12.3-fold compared to control, respectively (Fig. 1). In contrast, KCl, ACTH, AII, and dbcAMP induced 52.9-fold, 1.5-fold, 107.9-fold, and 15.6-fold compared to control, respectively in the stable *CYP11B2*-H295R cells (Fig. 1). Therefore, although the effects of ACTH and dbcAMP on *CYP11B2* transcription were almost identical between both cells, the responsiveness to KCl and AII were dramatically augmented in the stable *CYP11B2*-H295R cells.

Differential effects of ARBs on CYP11B2 *transcription in the presence or absence of AII*

I next examined the effects of various ARBs on *CYP11B2* transcription either in the presence or absence of AII using the stable *CYP11B2*-H295R cells. As shown in Fig. 2A, all ARBs including telmisartan, valsartan, losartan, and olmesartan suppressed the AII-induced *CYP11B2* transcriptional activation approximately in a dose dependent manner. In contrast, in the absence of AII, telmisartan at 10 μmol/L significantly increased *CYP11B2* transcription (Fig. 2B). These data indicate that telmisartan, in contrast to other ARBs, induces *CYP11B2* transcriptional activity in the absence of AII.

Time course/dose-response analyses of the telmisartan effects on the CYP11B2 *transcriptional activation*

I next performed time course and dose-response analyses of the telmisartan effects using the stable *CYP11B2*-H295R cells. As shown in Fig. 3A, telmisartan at 10 μmol/L induced *CYP11B2* transcriptional activation maximal at 12 h. Dose response analyses revealed that telmisartan dose-dependently increased *CYP11B2* transcriptional activity (Fig. 3B).

Stimulatory effects of telmisartan on CYP11B2 *mRNA expression/aldosterone secretion*

I next examined the effects of telmisartan on *CYP11B2* mRNA expression and aldosterone secretion. Time course analyses of *CYP11B2* mRNA expression revealed that telmisartan at 10 μmol/L increased *CYP11B2* mRNA expression maximal at 12 h (Fig. 4A) in H295R cells as well as *CYP11B2* transcriptional activation (Fig. 3A). As shown in Fig. 4B, telmisartan also increased *CYP11B2* mRNA expression dose-dependently in H295R cells as well as *CYP11B2* transcriptional activation (Fig. 3B). Additionally, telmisartan dose-dependently increased aldosterone secretion from H295R cells into the media (Fig. 4C). Taken together, telmisartan-induced *CYP11B2* transcriptional activation increases *CYP11B2* mRNA expression, which most likely results in the increase of aldosterone production/secretion.

Possible involvement of the Ad5 element in the telmisartan-mediated CYP11B2 *transcriptional activation*

I next examined the element responsible for the telmisartan-mediated *CYP11B2* transcriptional activation. As shown in Fig. 5A, the telmisartan effect was observed in *CYP11B2* 5'-flanking region deletion mutants from -1521/+2-luc to -135/+2-luc, but not in -106/+2-luc and -65/+2-luc. Since the Ad5 element is known to locate between -129 and -114,[5)] I used -1521/+2-luc-Ad5-mut whose Ad5 element was mutated. As shown in Fig. 5B, mutation of the element completely abrogated the telmisartan effect. These data indicate that telmisartan stimulates *CYP11B2* transcription via the Ad5 element.

Effects of telmisartan on mRNA expression of transcription factors involved in the CYP11B2 *transcription via binding to the Ad5 element*

I next examined the effects of telmisartan (10 μmol/L) on mRNA expression of transcription factors involved in the *CYP11B2* transcription via binding to the Ad5 element using the same RNAs used in Fig. 4A (*CYP11B2* mRNA time course analyses). Interestingly, telmisartan treatment induced both NGFIB (Fig. 6A) and NURR1 (Fig. 6B) mRNA expression maximal at 1 h, while gradually decreased SF-1 mRNA expression (Fig. 6C). In contrast, mRNA expression of chicken ovalbumin upstream promoter transcription factor (COUP-TF), which is also known to bind to the Ad5 element,[5] was not affected (data not shown). Since NGFIB[8] and NURR1[8] are both known to trans-activate and SF-1[8, 9] is known to trans-repress the Ad5 element, the increase of NGFIB and NURR1 and the decrease of SF-1 may be involved in the telmisartan effect.

Possible involvement of CaMK-mediated NURR1 induction in the telmisartan effect

Since mRNA expression of NGFIB and NURR1 is known to be induced by AII,[8] and the induction was reported to be mediated via CaMK, protein kinase C (PKC), and Src tyrosine kinase (SRC),[10] I next examined the involvement of these kinases in the telmisartan effect. When KN-93,[7] a CaMK inhibiter, was co-incubated, the effect of telmisartan on *CYP11B2* transcription was completely abrogated, while a PKC inhibitor

Ro-31-8220[11] and a SRC inhibitor Src-I1[10] did not affect it (Fig. 7A). I therefore examined the involvement of CaMK on the mRNA expression of *CYP11B2*, NGFIB, and NURR1. Since telmisartan induced the mRNA expression of *CYP11B2* (Fig. 4A) maximally at 12 h, and NGFIB (Fig. 6A) and NURR1 (Fig. 6B) maximally at 1 h, we examined the effects of KN-93 at the indicated time points. As shown in Fig 7B, *CYP11B2* mRNA expression at 12 h was almost completely abrogated by KN-93 treatment. Although KN-93 treatment did not affect NGFIB mRNA expression at 1 h (Fig. 7C), it almost completely abrogated NURR1 mRNA expression at 1 h (Fig. 7D). Moreover, over-expression of NURR1, but not NGFIB, further augmented the telmisartan-mediated *CYP11B2* transcriptional activation (Fig. 7E). These data indicate that CaMK-mediated NURR1 induction may be involved in the telmisartan effect.

AII type 1 receptor may not be involved in the telmisartan effect

In order to examine the possible involvement of AII type 1 receptor (AT1R) in the telmisartan effect, I co-incubated the *CYP11B2*-H295R cells with telmisartan and olmesartan (olmesartan was added to the media 3 h prior to telmisartan). As shown in Fig. 8, olmesartan did not affect the telmisartan-mediated *CYP11B2* transcription even at its high doses (Fig. 8). Therefore, AT1R may not be involved in the telmisartan effect.

DISCUSSION

Analyses of *CYP11B2* transcription have so far been examined using transient transfection experiments. However, since transient transfection is technically complicated and normalization by β-galactosidase activity is necessary each time, using a stable cell line is favorable. I here have first generated a stable cell line expressing *CYP11B2* 5'-flanking region/luciferase cDNA chimeric plasmid (*CYP11B2*-H295R cells). The cell line has demonstrated a significant responsiveness of *CYP11B2* transcription to both AII (107.9-fold) and potassium (52.9-fold) in contrast to transient transfection analyses (AII: 5.7-fold, potassium: 8.5-fold) (Fig. 1). Consistently, in the previous studies, responsiveness of *CYP11B2* to AII was between 2- to 14.5-fold[7), 12-16)] and that to potassium was between 2.5- to 6.5-fold[7), 13), 14), 16)] by transient transfection. Although the mechanism(s) for the significant responsiveness of *CYP11B2* to AII and potassium in the stable *CYP11B2*-H295R cells remains uncertain, the position in the chromosomes where the chimeric plasmid was integrated may possibly be important. The cells may therefore be useful for the innovation of novel anti-hypertensive therapy/drugs that inhibit AII- or potassium-mediated *CYP11B2* transcriptional activation.

I thereafter compared the effects of various ARBs on *CYP11B2* transcription using the stable *CYP11B2*-H295R cells. As expected, all ARBs inhibited the AII-mediated *CYP11B2* transcriptional activation (Fig. 2A), and the inhibition of *CYP11B2* transcription may possibly result in the decrease of aldosterone secretion. Since aldosterone is well known to induce damages in organs such as brain, heart, and kidney,[7] the ARB-mediated *CYP11B2* transcriptional inhibition may ameliorate these organ damages. However, in the absence of AII, telmisartan, but not other ARBs, stimulated *CYP11B2* transcription (Fig. 2B, Fig. 3) as well as *CYP11B2* mRNA expression and aldosterone secretion (Fig. 4). These data indicate that careful attention is needed for choosing ARBs depending on patient situations such as endogenous AII levels. Among ARBs, telmisartan is specific in that it functions as partial agonist of PPAR-γ.[17] I therefore co-incubated the *CYP11B2*-H295R cells with telmisartan and PPAR-γ antagonist GW9662 (GW9662 was added to the media 30 min prior to telmisartan).[7] However, GW9662 did not inhibit, but rather slightly stimulated the telmisartan-mediated *CYP11B2* transcriptional activation (data not shown). This is consistent to our previous observation regarding the inhibitory effects of PPAR-γ agonist on the AII-mediated *CYP11B2* transcriptional activation.[7] Taken together, it is suggested that PPAR-γ activation may not be involved in the telmisartan effect.

Transient transfection experiments using *CYP11B2* 5'-flanking region deletion mutants/Ad5 mutant have revealed that the Ad5 element was responsible for the telmisartan-mediated *CYP11B2* transcriptional activation (Fig. 5). Examinations of the effects of telmisartan on mRNA expression of transcription factors that are known to bind to the Ad5 element have shown that telmisartan induced the mRNA expression of positively-regulating NGFIB[8] and NURR1[8] at 1 h, while gradually decreased that of negatively-regulating SF-1[8, 9] (Fig. 6). Recently, AII was also demonstrated to induce NGFIB and NURR1 mRNA expression rapidly (~1 h) in H295R cells[10, 18] as well as in mice.[19] The AII-mediated effect was demonstrated to be mediated via CaMK, PKC, and SRC pathways.[10] Since these pathways are known to be involved in the AII-mediated *CYP11B2* transcriptional activation,[5, 20] I next examined the involvement of these pathways in the telmisatran effect. The telmisartan-mediated *CYP11B2* transcriptional activation was completely abrogated by KN-93, a CaMK inhibitor, treatment, but not by inhibitors of other kinases (Fig. 7A). Moreover, KN-93 treatment almost completely inhibited the mRNA expression of *CYP11B2* (Fig. 7B) and NURR1 (Fig. 7D). Since NGFIB mRNA expression was not affected by KN-93 treatment (Fig. 7C), and the over-expression of NGFIB little affected the telmisartan-mediated *CYP11B2* transcription (Fig. 7E), CaMK-mediated NURR1 induction may possibly be the main pathway of the telmisartan effect. Moreover, since I have observed an increase of

H295R cell intracellular Ca^{2+} by telmisarten treatment (data not shown), it is highly possible that both AII and telmisartam activate the same pathway from Ca^{2+}induction, CaMK activation, through NURR1 induction (Fig. 9).[10), 20)]

I also examined the possibility if telmisartan activates AT1R as its partial agonist, as telmisartan has a unique “delta lock” structure that is involved in its strong affinity to AT1R among ARBs.[21)] Since olmesartan was demonstrated to have the strongest inhibitory effect on the AII-mediated *CYP11B2* transcriptional activation (Fig. 2A), I co-incubated the *CYP11B2*-H295R cells with telmisartan and olmesartan (olmesartan was added to the media 3 h prior to telmisartan). Olmesartan, however, did not diminish the telmisartan-mediated *CYP11B2* transcriptional activation even at 50 μmol/L (Fig. 8). Therefore, it is speculated that AT1R activation may not be involved in the telmisartan effect. Since telmisartan is specific among ARBs as it contains a methylbenzimidazole ring structure, its structural specificity may contribute to the effect. Further studies are needed to clarify the whole signaling pathway of the telmisartan effect, and their information may give us clues for the innovation of novel anti-hypertensive therapy/drugs.

CONCLUSION

I here generated a stable H295R cell line expressing *CYP11B2* 5'-flanking region/luciferase cDNA chimeric construct that is highly sensitive to AII and KCl. Using the cell line, I observed that telmisartan, but not other ARBs, induced *CYP11B2* transcriptional activity in the absence of AII. Activation of CaMK as well induction of NURR1 may at least be involved in the telmisartan-mediated *CYP11B2* transcriptional activation.

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FIGURE LEGENDS

Figure 1. Effects of KCl, ACTH, AII, and dbcAMP on *CYP11B2* transcription. Either H295 cells transiently transfected with -1521/+2-luc and pCMV-β-gal or the stable *CYP11B2*-H295R cells were incubated without (control) or with 10.4 mmol/L KCl, 10 μmol/L ACTH, 100 nmol/L AII, or 1 mmol/L dbcAMP for 6 h. Open columns (transient), transiently transfected H295R cells. Closed columns (stable), the stable *CYP11B2*-H295R cells. Data represent mean ± SEM (n = 6), percent control.

Figure 2. Effects of various ARBs on *CYP11B2* transcription. (A) Effects of ARBs in the presence of AII. The stable *CYP11B2*-H295R cells were incubated either without or with indicated concentrations (μmol/L) of various ARBs including telmisartan (Tel), valsartan (Val), losartan (Los), and olmesartan (Olm) for 12 h. In some experiments, the cells were co-treated with AII (100 nmol/L) for the last 6 h. Data represent mean ± SEM (n = 6), percent control (the absence of both AII and ARB). (B) Effects of ARBs in the absence of AII. The stable *CYP11B2*-H295R cells were incubated either without or with indicated concentrations (μmol/L) of various ARBs including telmisartan (Tel), valsartan (Val), losartan (Los), and olmesartan (Olm) for 12 h. Data represent mean ± SEM (n = 6), percent control (the absence of ARB). * $P < 0.01$, *vs*. control.

Figure 3. Time course and dose-response analyses of the telmisartan effects. (A) Time

course analyses. The stable *CYP11B2*-H295R cells were incubated with telmisartan (10 μmol/L) for the indicated times (h). Data represent mean ± SEM (n = 4), percent control (time 0). * $P < 0.01$, *vs.* 0 h. (B) Dose-response analyses. The stable *CYP11B2*-H295R cells were incubated with telmisartan (12 h) for the indicated concentrations (μmol/L). Data represent mean ± SEM (n = 4), percent control (0 μmol/L). * $P < 0.01$, *vs.* 0 μmol/L.

Figure 4. Effects of telmisartan on *CYP11B2* mRNA expression/aldosterone secretion. (A) Time course analyses of *CYP11B2* mRNA expression. H295R cells were incubated with telmisartan (10 μmol/L) for the indicated times. Data represent mean ± SEM (n = 3), percent control (0 μmol/L). * $P < 0.05$, *vs.* 0 h. (B) Dose-response analyses of *CYP11B2* mRNA expression. H295R cells were incubated with telmisartan (12 h) for the indicated concentrations (μmol/L). Data represent mean ± SEM (n = 3), percent control (0 μmol/L). * $P < 0.01$, *vs.* 0 μmol/L. (C) Dose-response analyses of aldosterone secretion into the media. H295R cells were incubated with telmisartan (12 h) for the indicated concentrations (μmol/L). Data represent mean ± SEM (n = 4), percent control (0 μmol/L). * $P < 0.01$, *vs.* 0 μmol/L.

Figure 5. Effects of 5'-flanking region mutants on the telmisartan-mediated *CYP11B2* transcriptional activation. (A) Effects of *CYP11B2* 5'-flanking region deletion mutants.

Either -1521/+2-luc, -747/+2-luc, -135/+2-luc, -106/+2-luc, -65/+2-luc, or pGL3-Basic (control plasmid) was transiently transfected with pCMV-β-gal into H295R cells, and the cells were thereafter incubated without (open columns) or with 10 μmol/L (filled columns) telmisartan (Tel) for 12 h. Data represent mean ± SEM (n = 4-10), percent control (0 μmol/L in -1521/+2-luc). * $P < 0.01$, *vs.* 0 μmol/L in -1521/+2-luc. †$P < 0.01$, *vs.* 0 μmol/L in -747/+2-luc. ¶ $P < 0.01$, *vs.* 0 μmol/L in -135/+2-luc. (B) Effects of the Ad5 element mutant. Either -1521/+2-luc, -1521/+2-luc-Ad5-mut or pGL3-Basic (control plasmid) was transiently transfected with pCMV-β-gal into H295R cells, and the cells were thereafter incubated without (open columns) or with 10 μmol/L (filled columns) telmisartan (Tel) for 12 h. Data represent mean ± SEM (n = 4), percent control (0 μmol/L in -1521/+2-luc). * $P < 0.01$, *vs.* 0 μmol/L in -1521/+2-luc.

Figure 6. Effects of telmisartan on the mRNA expression of transcription factors involved in the *CYP11B2* transcriptional regulation via binding to the Ad5 element. (A) Time course analyses of NGFIB mRNA expression. H295R cells were incubated with telmisartan (10 μmol/L) for the indicated times. Data represent mean ± SEM (n = 3), percent control (0 μmol/L). * $P < 0.05$, *vs.* 0 h. (B) Time course analyses of NURR1 mRNA expression. H295R cells were incubated with telmisartan (10 μmol/L) for the indicated times. Data represent mean ± SEM (n = 3), percent control (0 μmol/L). * $P <$

0.01, *vs.* 0 h. (C) Time course analyses of SF-1 mRNA expression. H295R cells were incubated with telmisartan (10 μmol/L) for the indicated times. Data represent mean ± SEM (n = 3), percent control (0 μmol/L). In (A)-(C), used RNAs were identical to those used in Fig. 4A.

Figure 7. Effects of protein kinase inhibitors and NURR1/NGFIB over-expression on the telmisartan-mediated effects. (A) Effects of KN-93, Ro-31-8220, and Src-11 on the telmisartan-mediated *CYP11B2* transcriptional activation. The stable *CYP11B2*-H295R cells were incubated either without or with 10 μmol/L telmisartan (Tel) for 12 h in the absence or presence of 5 μmol/L protein kinase inhibitors (the inhibitors were added to the media 30 min prior to telmisartan addition). Data represent mean ± SEM (n = 4), percent control (in the absence of both inhibitors and telmisartan). * $P < 0.05$, *vs.* 10 μmol/L telmisartan without inhibitors. (B) Effects of KN-93 on the telmisartan-mediated *CYP11B2* mRNA expression. H295R cells were incubated with 10 μmol/L telmisartan (Tel) for 12 h in the absence or presence of 5 μmol/L KN-93 (KN-93 was added to the media 30 min prior to telmisartan addition). Data represent mean ± SEM (n = 4), percent control (in the absence of both KN-93 and telmisartan). * $P < 0.01$, *vs.* 10 μmol/L telmisartan without KN-93. (C) Effects of KN-93 on the telmisartan-mediated NGFIB mRNA expression. H295R cells were incubated with 10

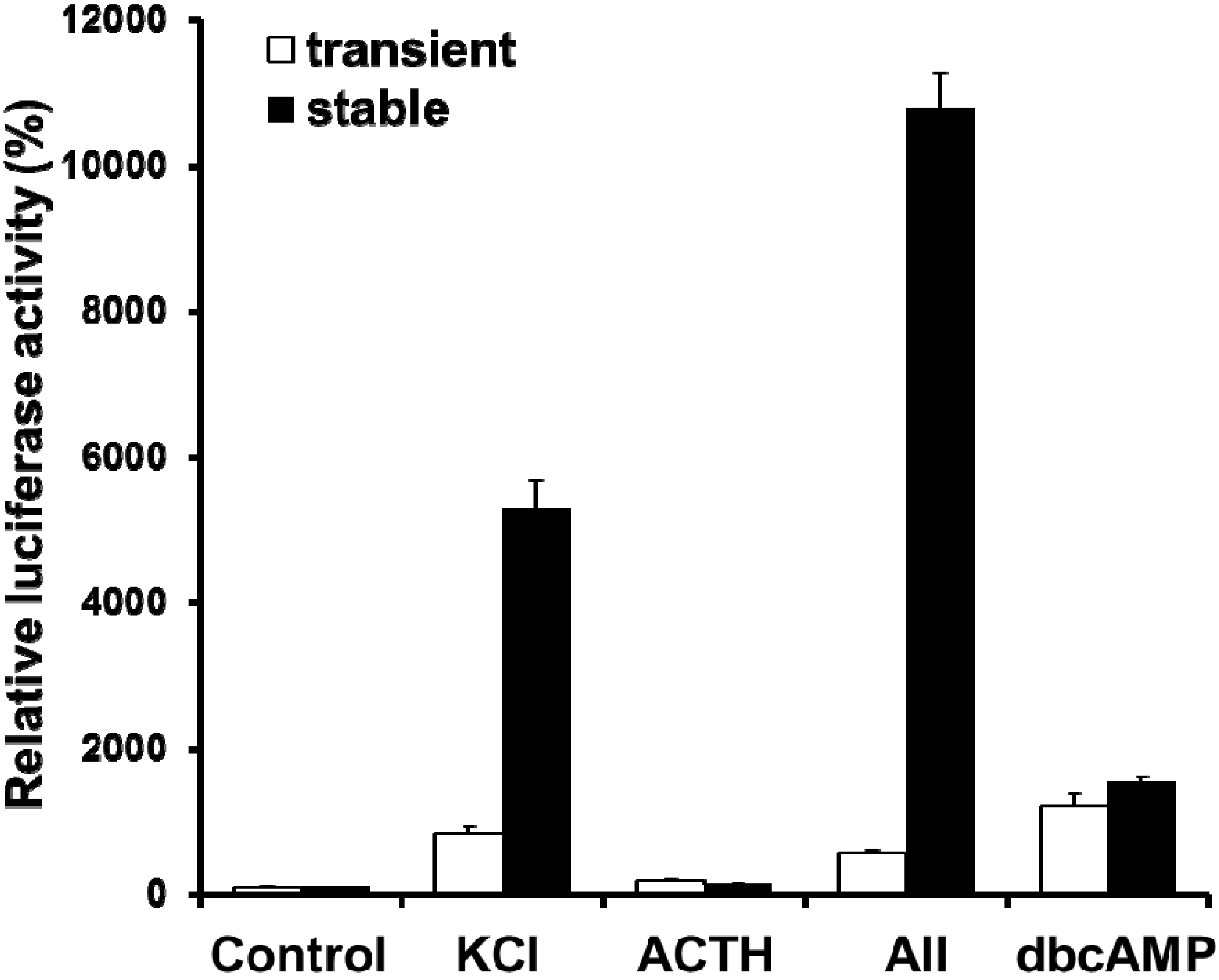
μmol/L telmisartan (Tel) for 1 h in the absence or presence of 5 μmol/L KN-93 (KN-93 was added to the media 30 min prior to telmisartan addition). Data represent mean ± SEM (n = 4), percent control (in the absence of both KN-93 and telmisartan). (D) Effects of KN-93 on the telmisartan-mediated NURR1 mRNA expression. H295R cells were incubated with 10 μmol/L telmisartan (Tel) for 1 h in the absence or presence of 5 μmol/L KN-93 (KN-93 was added to the media 30 min prior to telmisartan addition). Data represent mean ± SEM (n = 4), percent control (in the absence of both KN-93 and telmisartan). * $P < 0.01$, *vs.* 10 μmol/L telmisartan without KN-93. (E) Effects of NGFIB/NURR1 over-expression. H295R cells were transfected with either -1521/+2-luc and pcDNA3 (control plasmid), -1521/+2-luc and pcDNA3-NGFIB, or -1521/+2-luc and pcDNA3-NURR1 with pCMV-β-gal, and were thereafter incubated without or with 10 μmol/L telmisartan (Tel) for 12 h. Data represent mean ± SEM (n = 4), percent control (0 μmol/L in -1521/+2-luc plus pcDNA3). * $P < 0.01$, *vs.* 10 μmol/L in -1521/+2-luc plus pcDNA3.

Figure 8. Effects of olmesartan on the telmisartan-mediated *CYP11B2* transcriptional activation. The stable *CYP11B2*-H295R cells were incubated either without or with 10 μmol/L telmisartan (Tel) for 12 h in the absence or presence of indicated concentrations (μmol/L) of olmesartan (Olm) (olmesartan was added to the media 3 h prior to

telmisartan). Data represent mean ± SEM (n = 4), percent control (in the absence of both olmesartan and telmisartan).

Figure 9. The possible mechanism of the telmisartan-mediated *CYP11B2* transcriptional activation.

Figure 1



transient
stable
Relative luciferase activity (%)
12000
10000
8000
6000
4000
2000
0
Control
KCl
ACTH
AII
dbcAMP

Figure 2

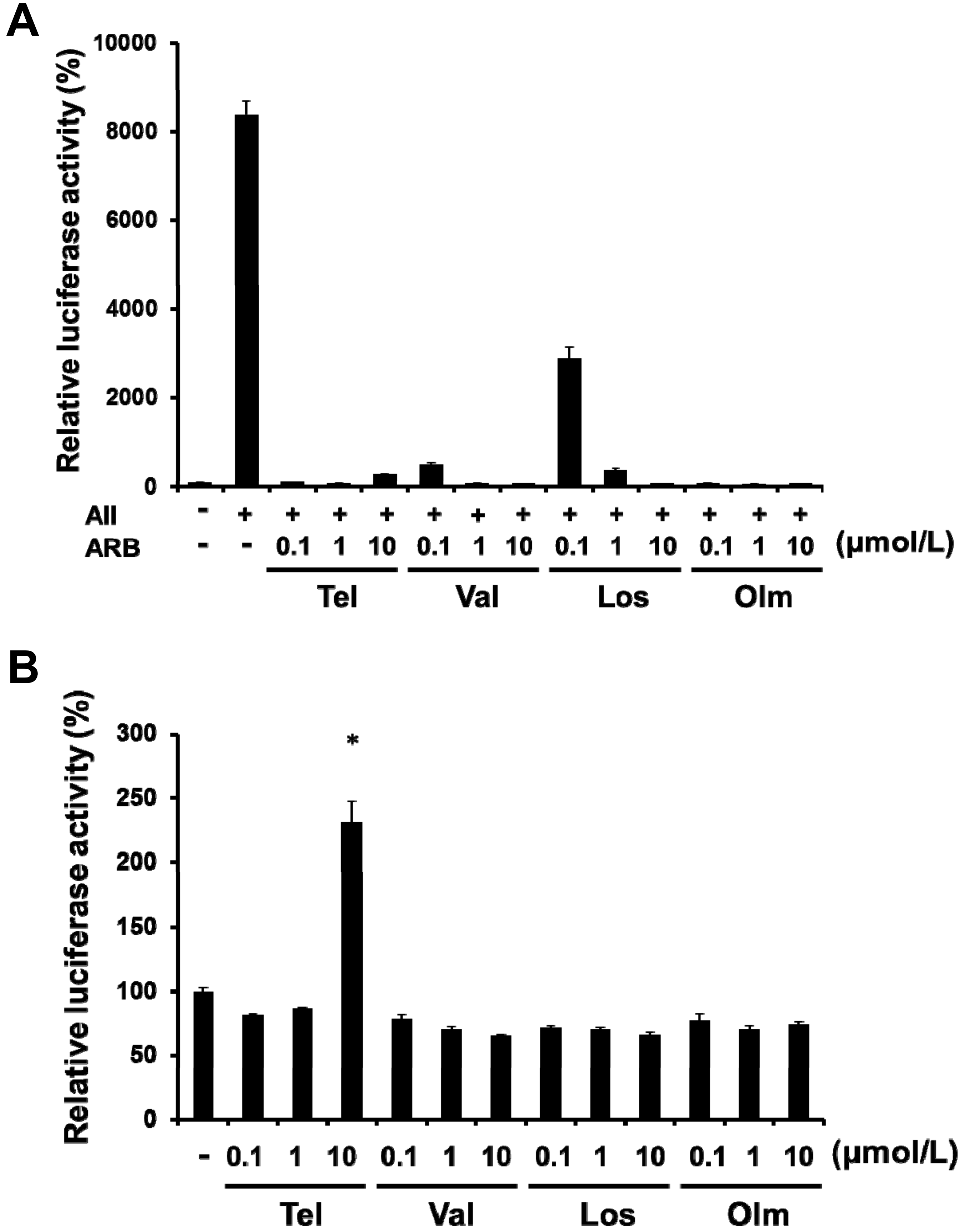

A
Relative luciferase activity (%)
10000
8000
6000
4000
2000
0
AII - + + + + + + + + + + + + +
ARB - - 0.1 1 10 0.1 1 10 0.1 1 10 0.1 1 10 (μmol/L)
Tel
Val
Los
Olm
B
Relative luciferase activity (%)
300
250
200
150
100
50
0
*
- 0.1 1 10 0.1 1 10 0.1 1 10 0.1 1 10 (μmol/L)
Tel
Val
Los
Olm

Figure 3

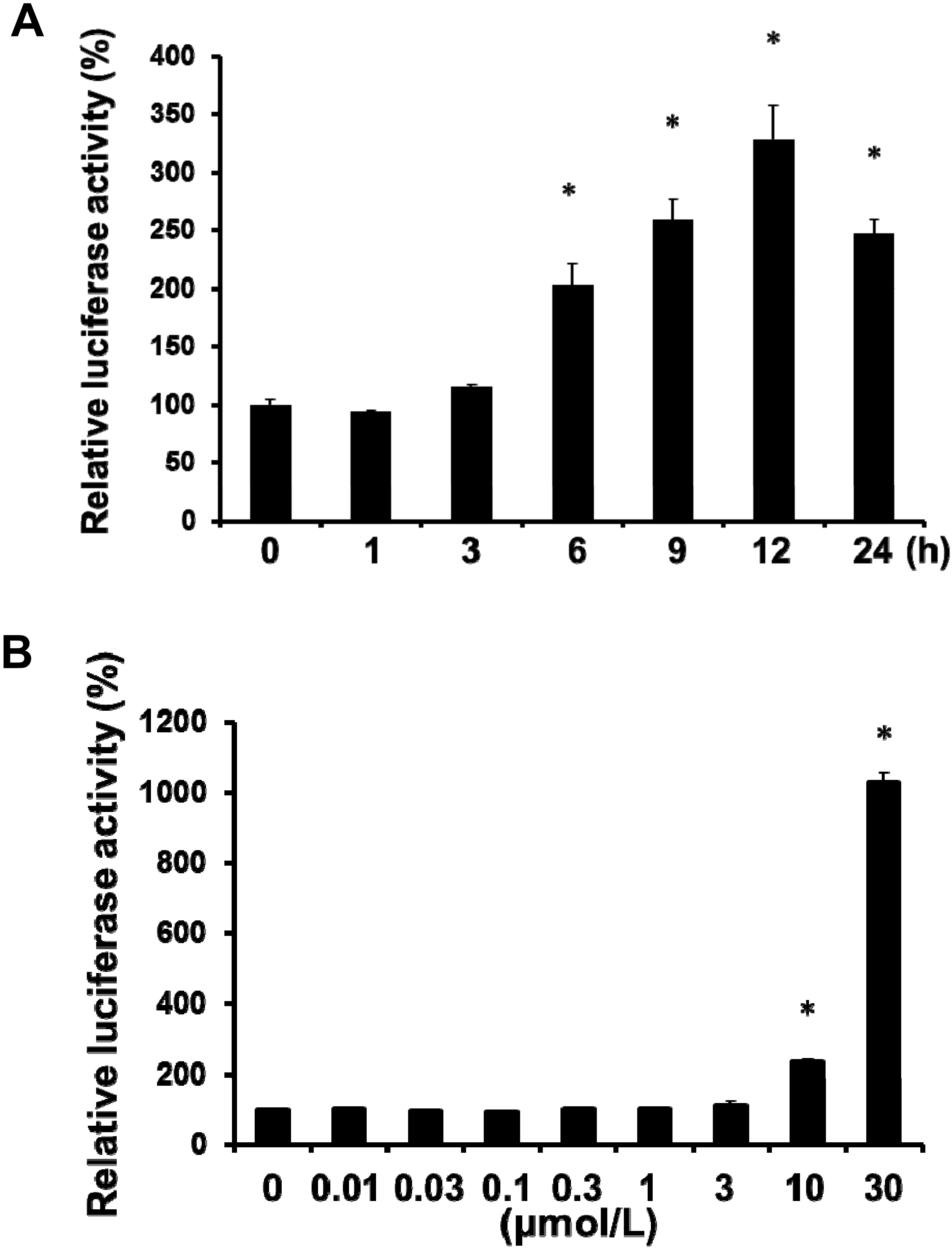
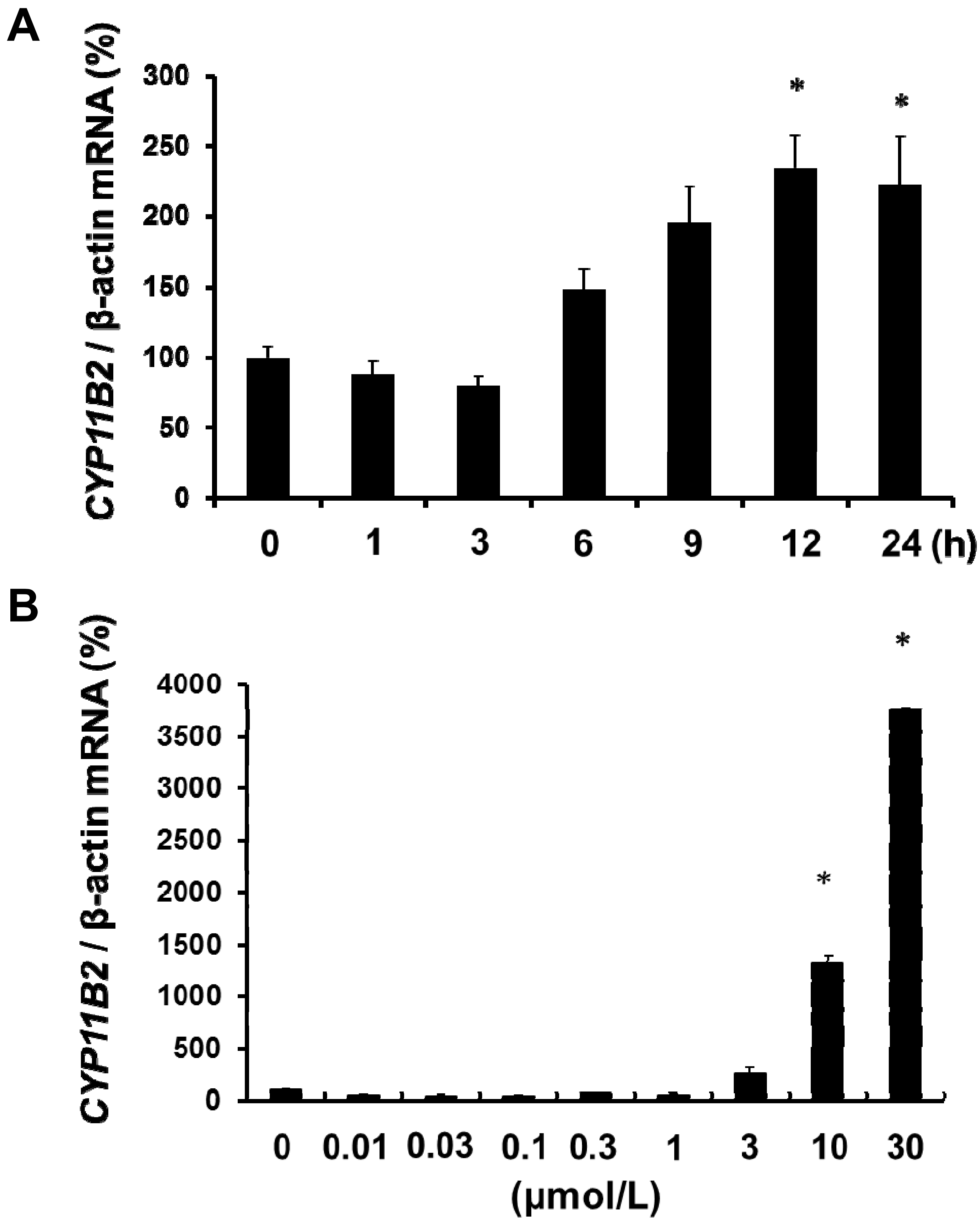

A
Relative luciferase activity (%)
400
350
300
250
200
150
100
50
0
*
*
*
*
0
1
3
6
9
12
24 (h)
B
Relative luciferase activity (%)
1200
1000
800
600
400
200
0
*
*
0
0.01
0.03
0.1
0.3
1
3
10
30
(µmol/L)

Figure 4



A
CYP11B2 / β-actin mRNA (%)
300
250
200
150
100
50
0
*
*
0
1
3
6
9
12
24 (h)
B
CYP11B2 / β-actin mRNA (%)
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
0
0.01
0.03
0.1
0.3
1
3
10
30
(μmol/L)

Figure 4

C

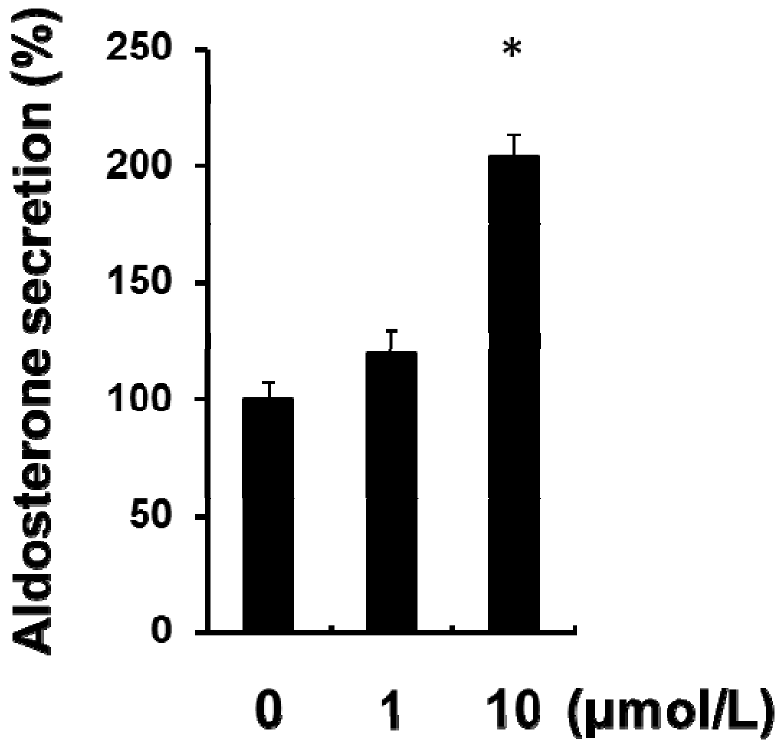


Figure 5

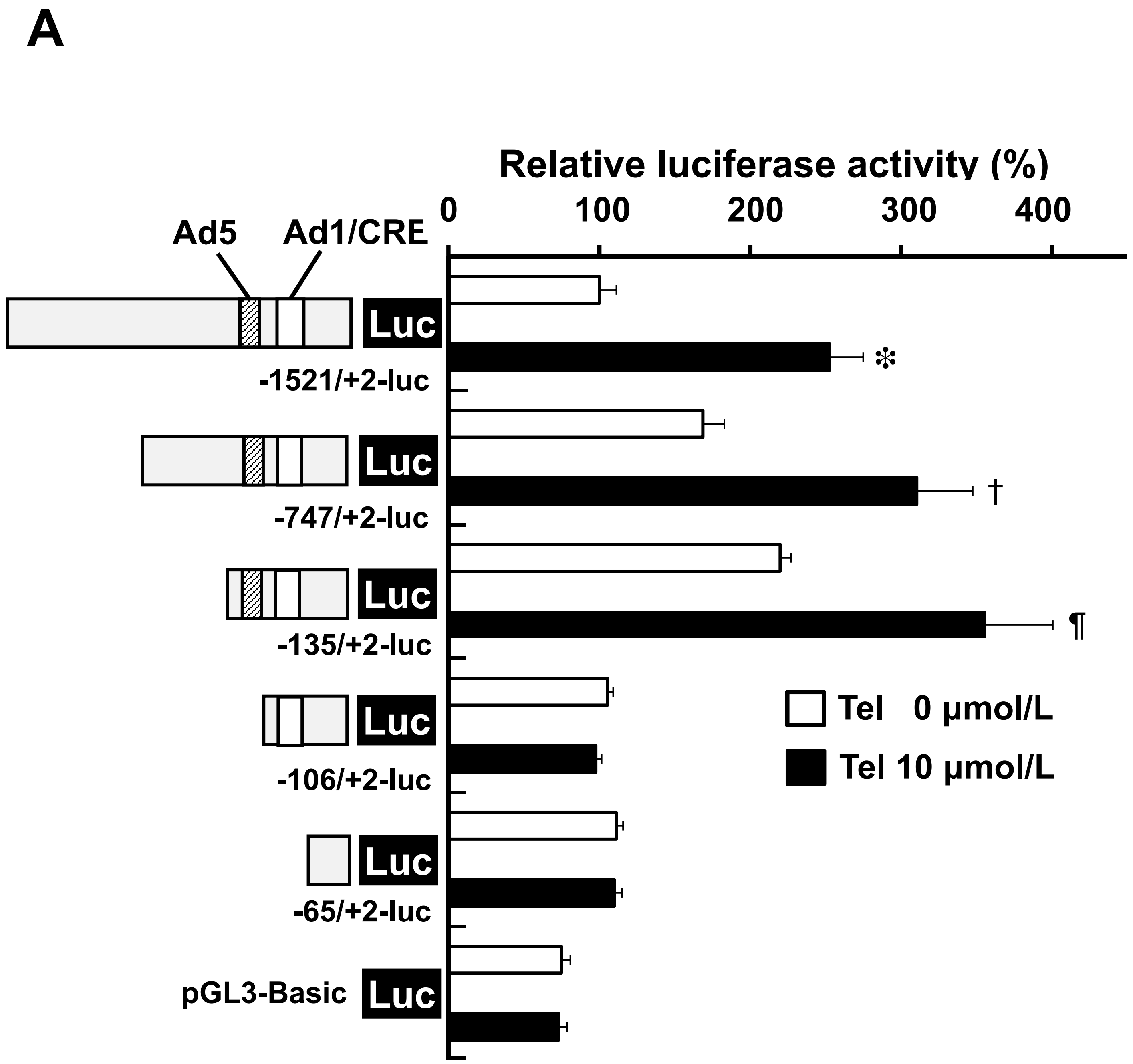

A
Relative luciferase activity (%)
0
100
200
300
400
Ad5
Ad1/CRE
Luc
-1521/+2-luc
*
-747/+2-luc
†
-135/+2-luc
¶
-106/+2-luc
-65/+2-luc
pGL3-Basic
Tel 0 μmol/L
Tel 10 μmol/L

Figure 5

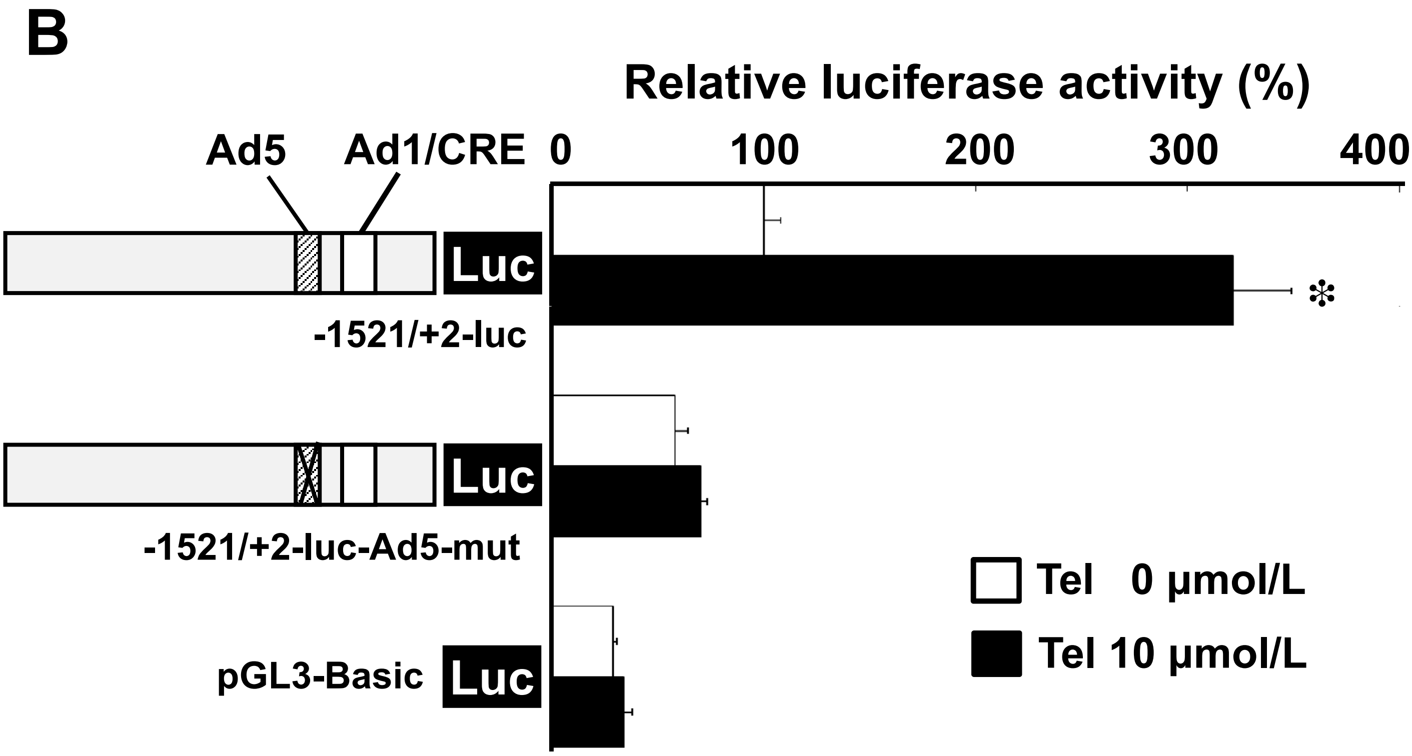
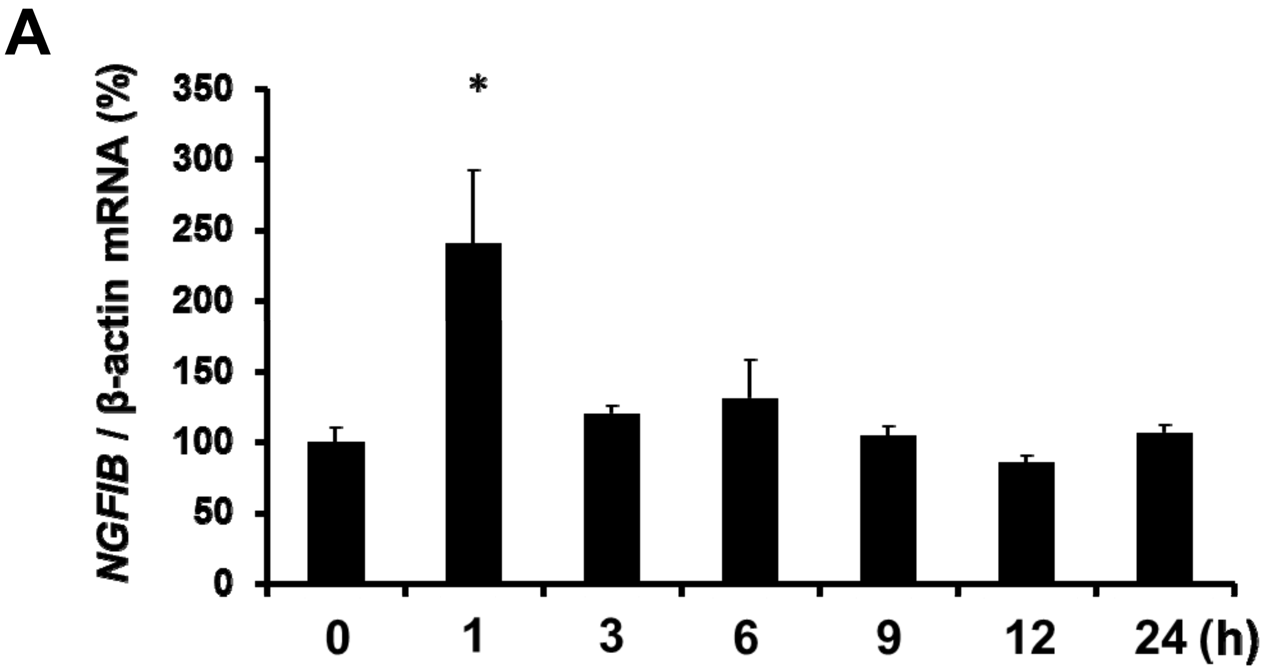
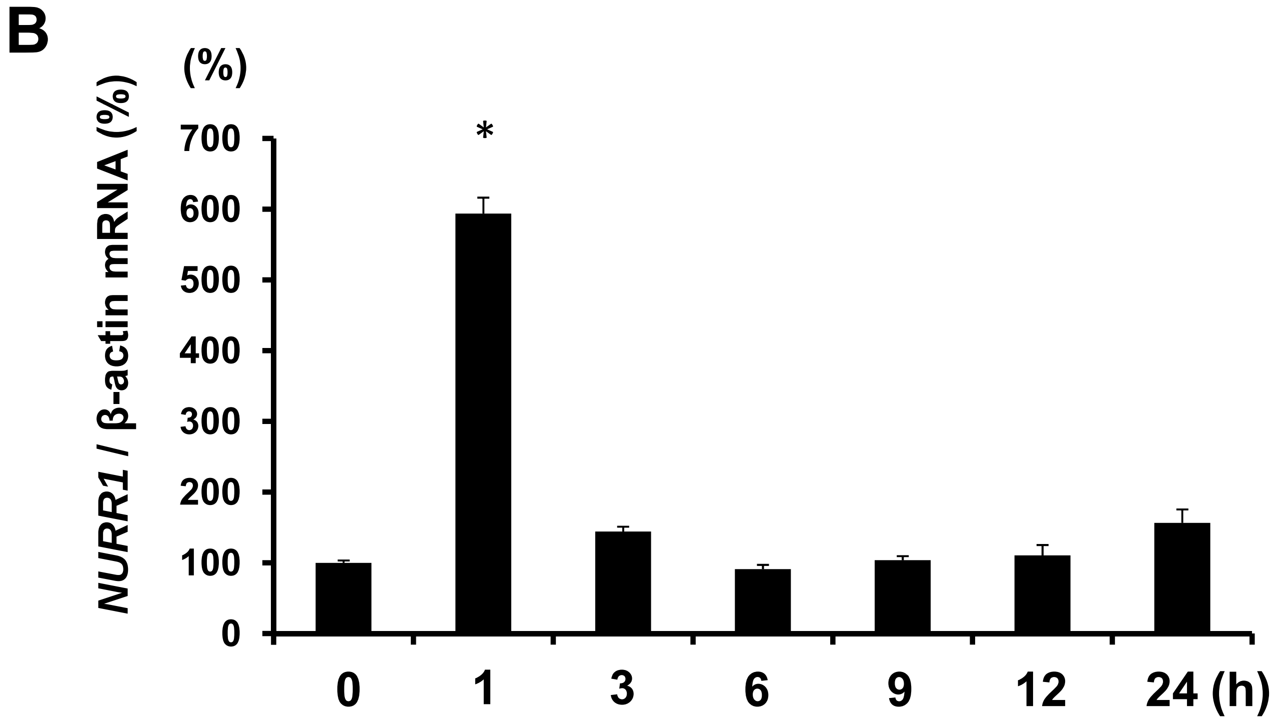

B
Relative luciferase activity (%)
Ad5
Ad1/CRE
0
100
200
300
400
Luc
-1521/+2-luc
*
Luc
-1521/+2-luc-Ad5-mut
Tel 0 μmol/L
Tel 10 μmol/L
pGL3-Basic
Luc

Figure 6



A
NGFIB / β-actin mRNA (%)
350
300
250
200
150
100
50
0
*
0
1
3
6
9
12
24 (h)



B
(%)
NURR1 / β-actin mRNA (%)
700
600
500
400
300
200
100
0
*
0
1
3
6
9
12
24 (h)

Figure 6

C

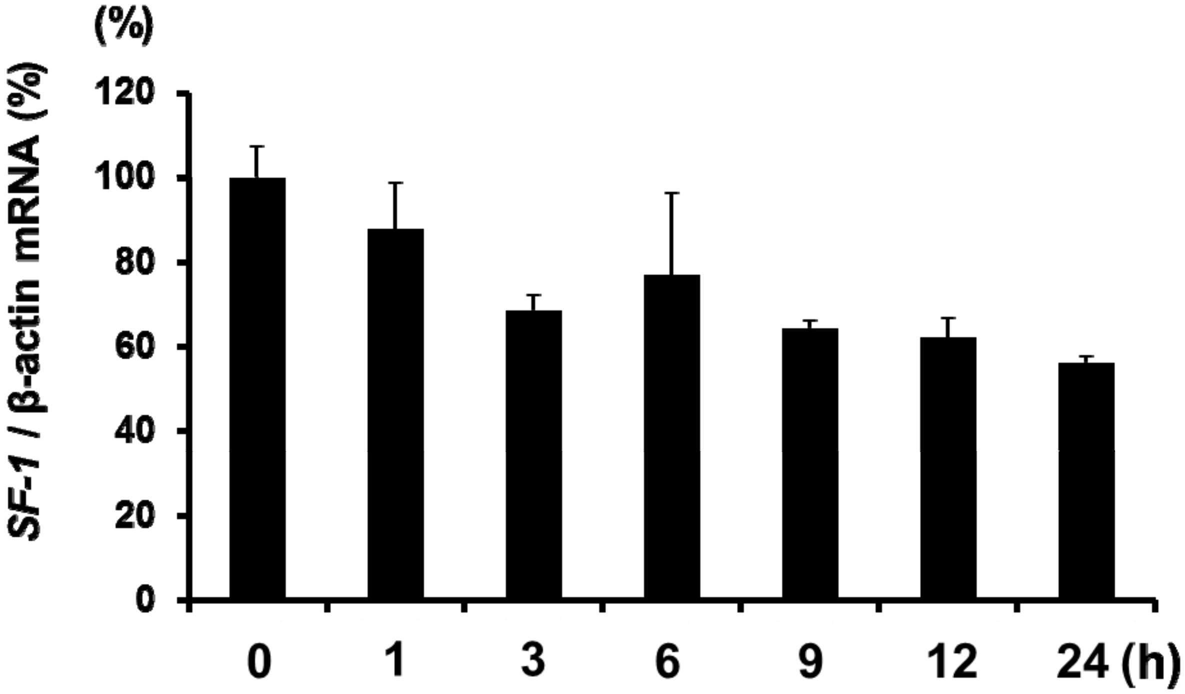


Figure 7

A

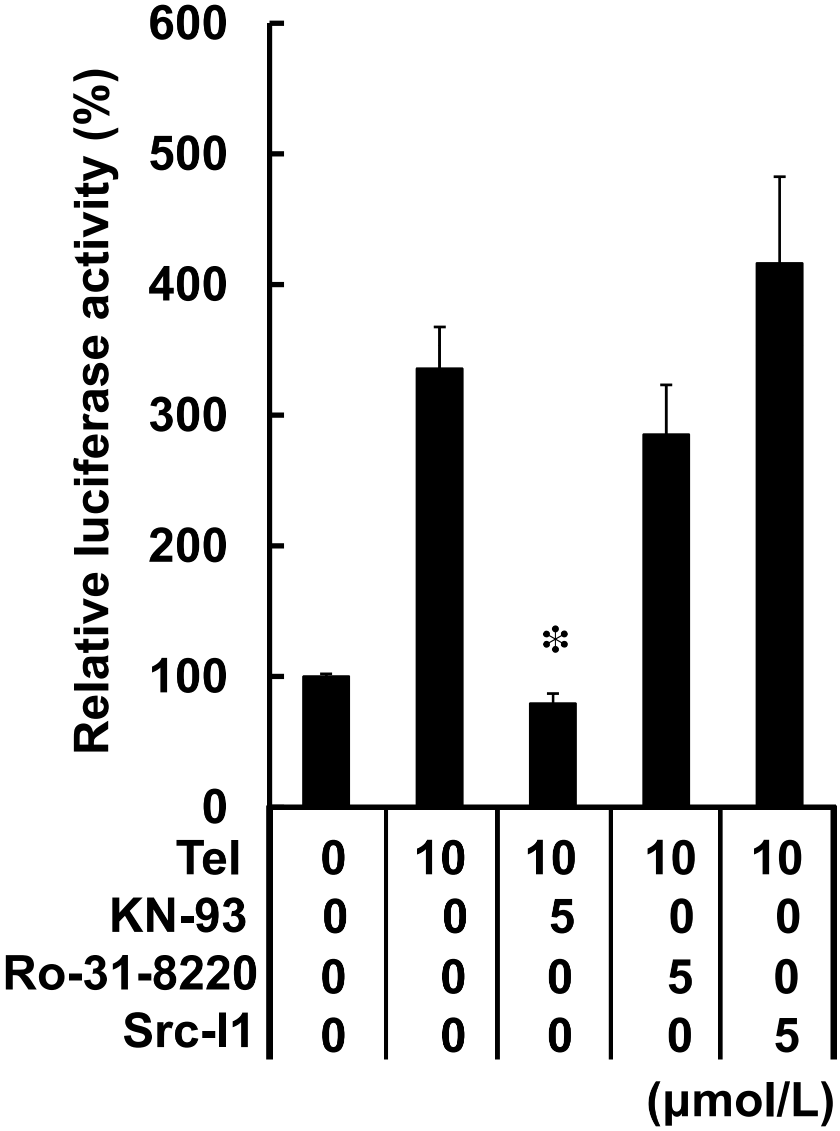
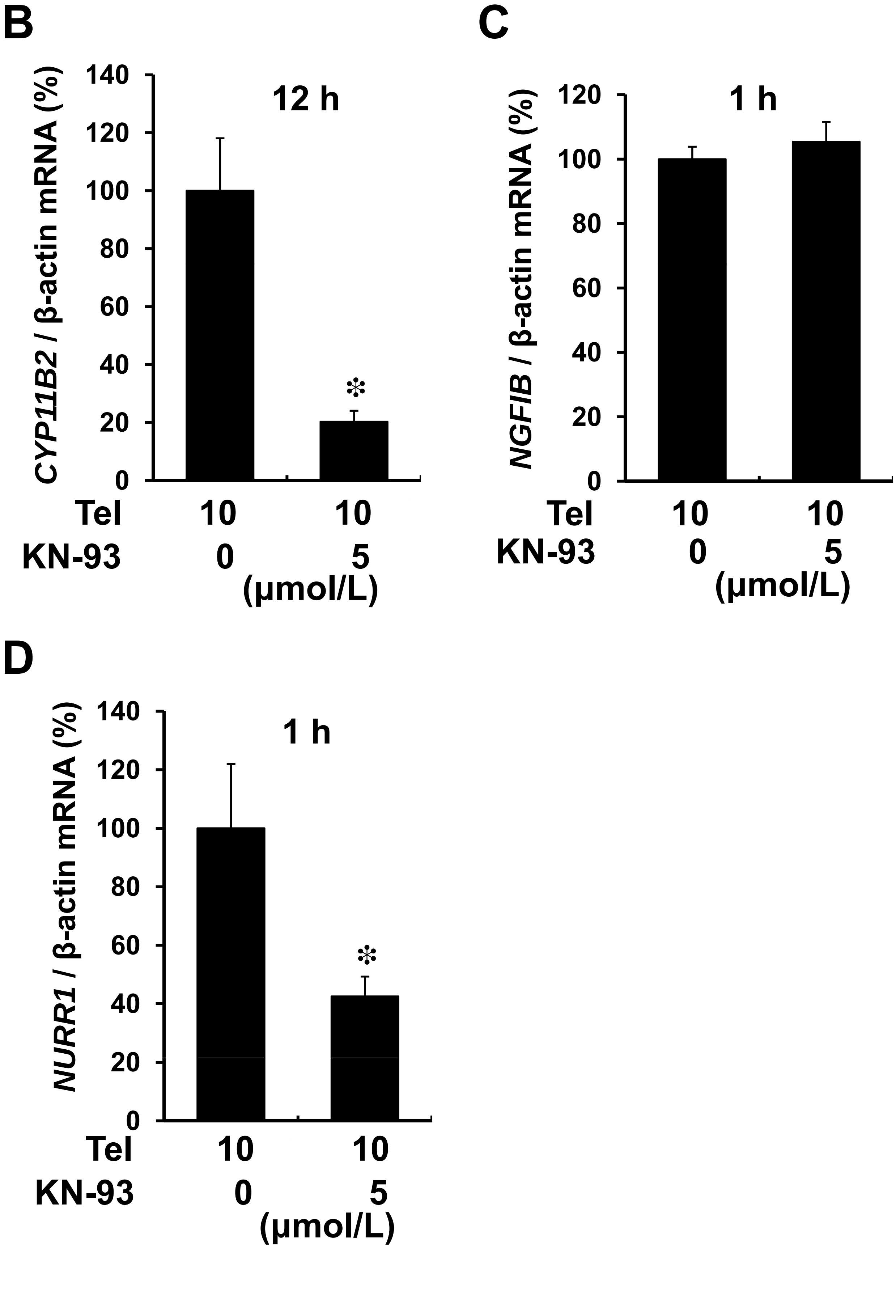

600
500
400
300
200
100
0
Relative luciferase activity (%)
*
Tel 0 10 10 10 10
KN-93 0 0 5 0 0
Ro-31-8220 0 0 0 5 0
Src-I1 0 0 0 0 5
(µmol/L)

Figure 7



B
12 h
CYP11B2 / β-actin mRNA (%)
0
20
40
60
80
100
120
140
*
Tel 10 10
KN-93 0 5
(μmol/L)
C
1 h
NGFIB / β-actin mRNA (%)
0
20
40
60
80
100
120
Tel 10 10
KN-93 0 5
(μmol/L)
D
1 h
NURR1 / β-actin mRNA (%)
0
20
40
60
80
100
120
140
*
Tel 10 10
KN-93 0 5
(μmol/L)

Figure 7

E

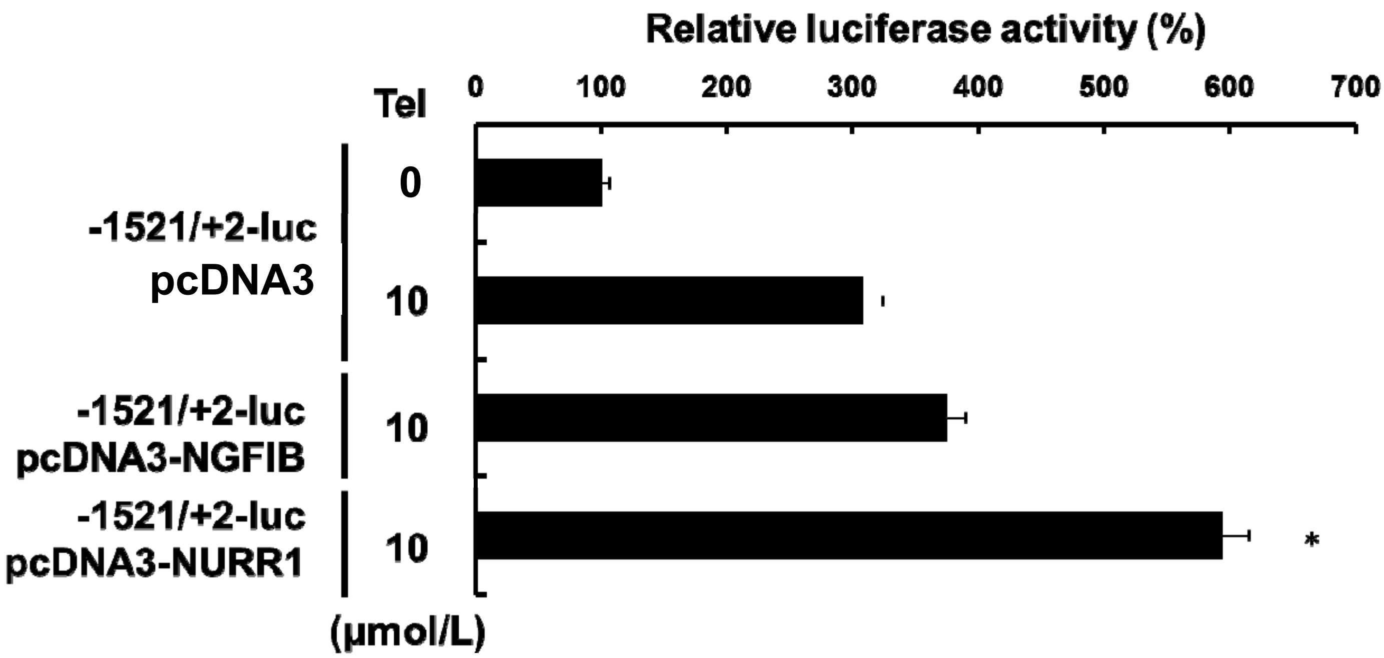
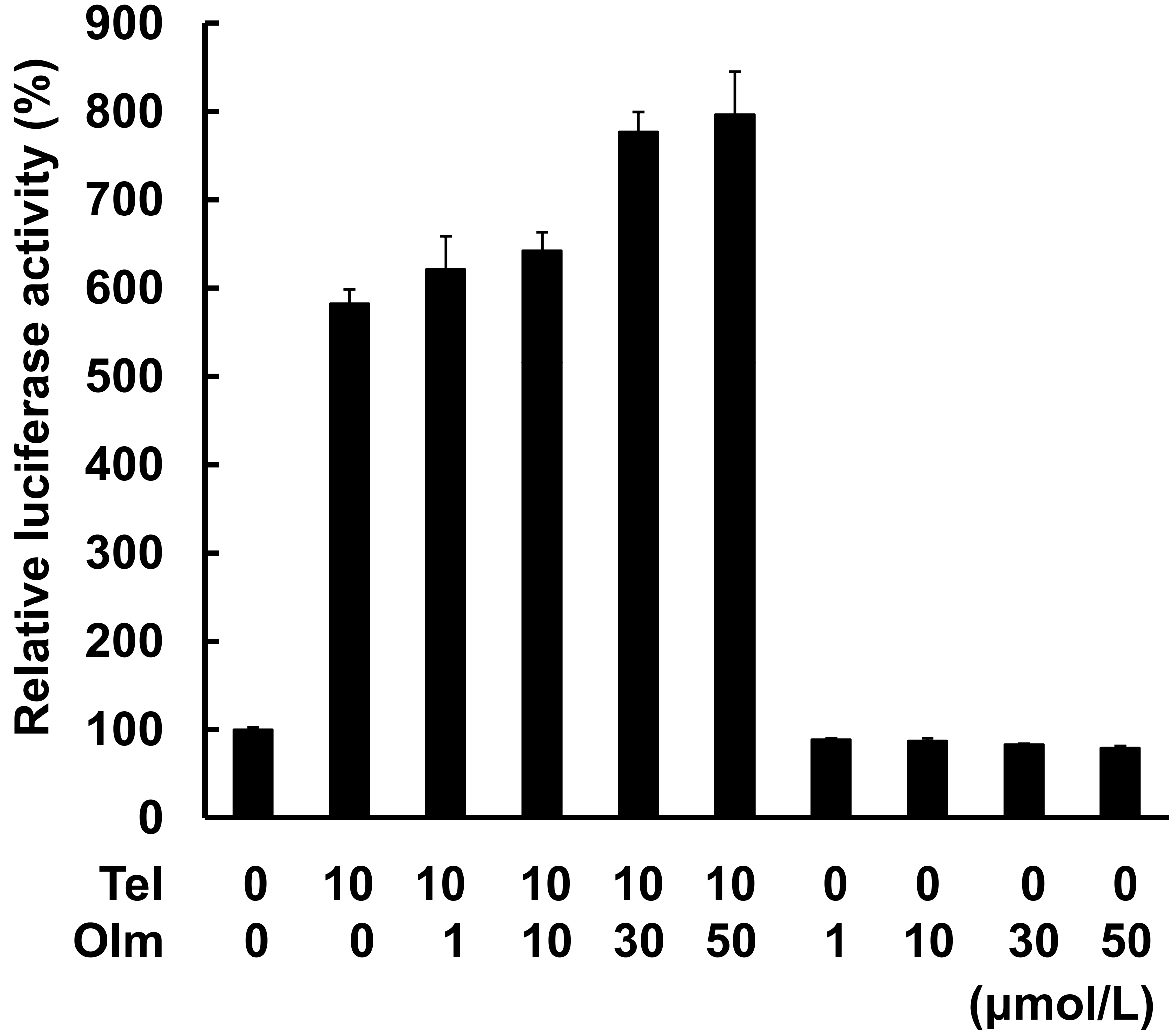

Relative luciferase activity (%)
0
100
200
300
400
500
600
700
Tel
0
-1521/+2-luc
pcDNA3
10
-1521/+2-luc
pcDNA3-NGFIB
10
-1521/+2-luc
pcDNA3-NURR1
10
*
(μmol/L)

Figure 8



900
800
700
600
500
400
300
200
100
0
Relative luciferase activity (%)
Tel 0 10 10 10 10 10 0 0 0 0
Olm 0 0 1 10 30 50 1 10 30 50
(μmol/L)

Figure 9